1 **New Tricks for an old molecule: Preserved antibacterial activity of** 2 **ribosomal protein S15 during evolution**

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23 **Abstract**

24 Previous studies show that some ribosomal proteins possess antimicrobial
 25 peptide (AMP) activity. However, information as such remains rather
 26 fragmentary and limited. Here we demonstrated for the first time that
 27 amphioxus RPS15, BjRPS15, was a previously uncharacterized AMP,
 28 which was not only capable of identifying Gram-negative and -positive
 29 bacteria via interaction with LPS and LTA but also capable of killing the
 30 bacteria. We also showed that both the sequence and 3D structure of
 31 RPS15 and its prokaryotic homologs were highly conserved, suggesting
 32 its antibacterial activity is universal across widely separated taxa.
 33 Actually this was supported by the facts that the residues positioned at
 34 45-67 formed the core region for the antimicrobial activity of BjRPS15,
 35 and its prokaryotic counterparts, including
 36 *Nitrospirae* RPS19₃₃₋₅₅, *Aquificae* RPS19₃₃₋₅₅ and *P. syringae* RPS19₅₀₋₇₂,
 37 similarly displayed antibacterial activities. BjRPS15 functioned by both
 38 interaction with bacterial membrane via LPS and LTA and membrane
 39 depolarization as well as induction of intracellular ROS. Moreover, we

showed that RPS15 existed extracellularly in amphioxus, shrimp, zebrafish and mice, hinting it may play a critical role in systematic immunity in different animals. In addition, we found that neither BjRPS15 nor its truncated form BjRPS15₄₅₋₆₇ were toxic to mammalian cells, making them promising lead molecules for the design of novel peptide antibiotics against bacteria. Collectively, these indicate that RPS15 is a new member of AMP with ancient origin and high conservation throughout evolution.

Author summary

Ribosomal protein, a component of ribonucleoprotein particles, is traditionally known involved in protein synthesis in a cell. Here we demonstrated for the first time that amphioxus ribosomal protein 15 was a novel antibacterial protein, capable of recognizing Gram-negative and -positive bacteria as well as killing them. It killed the bacteria by a combined mode of action of disrupting bacterial membrane integrity and inducing radical oxygen species production. We also showed that both eukaryotic ribosomal protein 15 and its prokaryotic counterpart ribosomal protein 19 possessed antibacterial activity, indicating that the antibacterial property is universal for this family of molecules. Moreover, we found that ribosomal protein 15 was present in the circulation system of various animals including shrimp, amphioxus, zebrafish and mice, suggesting it

may physiologically play a key role in systematic immunity. Altogether, our study provides a new angle for understanding the biological function of ribosomal proteins.

Introduction

The ribosome is an organelle within the cytoplasm of living cells that is composed of proteins and ribosomal RNAs (rRNAs), serving as the site for assembly of polypeptides encoded by messenger RNAs (mRNAs). Ribosomes are found in both prokaryotic and eukaryotic cells. In both types of cells, ribosomes are composed of two subunits, one large and one small [1,2]. Each subunit has its own mix of proteins and rRNAs. The small and large subunits of eukaryotes are called 40S and 60S, respectively, while those of prokaryotes called 30S and 50S, separately. Ribosomal protein S15 (RPS15) is a component of the 40S subunit of eukaryotes, while its homolog in prokaryotes is S19 (RPS19) of the 30S subunit [3].

Ribosomal proteins, in addition to their conventional role in ribosome assembly and protein translation, are shown involved in diverse physiological and pathological processes, such as neurodegeneration in Parkinson's disease, tumorigenesis, immune signaling and development [1,4,5]. Intriguingly, ribosomal proteins also show antimicrobial activity. Initially, the antimicrobial peptide (AMP) cecropin, first isolated from the

82 moth *Hyalophora cecropia* [6-8], was mapped to the N-terminal region of
83 the 50S ribosomal protein L1 of the pathogen *Helicobacter pylori* [9,10].
84 Recently, the 50S ribosomal proteins L27 and L30 of the lactic acid
85 bacterium *Lactobacillus salivarius* were shown to possess antimicrobial
86 activity against *Streptococcus pyogenes*, *Streptococcus uberis* and
87 *Enterococcus faecium* [11]. Furthermore, antibacterial activity was also
88 observed for the 60S ribosomal protein L29 isolated from the gill of
89 pacific oyster *Crassostrea gigas* [12] and the 40S ribosomal protein S30
90 isolated from the skin of rainbow trout *Oncorhynchus mykiss* [13]. It is
91 thus clear that some ribosomal proteins of the small and large subunits of
92 both prokaryotic and eukaryotic ribosomes can function as AMP.
93 However, our information regarding ribosomal protein AMPs is rather
94 fragmentary and limited. Moreover, little is known about the mode of
95 action of ribosomal protein AMPs. In this study, we identified RPS15 of
96 amphioxus (*Branchiostoma japonicum*), BjRPS15, as a novel member of
97 AMP, and demonstrated that BjRPS15 executed its antimicrobial activity
98 by both the interaction with bacterial membrane via LPS and LTA and
99 membrane depolarization as well as production of intracellular ROS. We
100 also showed that the emergence of antimicrobial activity of RPS15 could
101 be traced to its prokaryotic homolog RPS19. This is the first report
102 showing that RPS15 and its prokaryotic homolog RPS19 function as an
103 AMP, much enriching our understanding of the biological activities of

ribosomal proteins.

Results

Sequence characteristics and genomic structure of BjRPS15

The open reading frame (ORF) of *BjRPS15* (GenBank accession number: XP_019635827) obtained was 444 bp long, which encoded a deduced protein of 147 amino acids with a calculated molecular weight of about 16.96 kDa and an isoelectric point (pI) of 10.31 (Fig 1A). Analysis by SignalP showed that the deduced protein had no signal peptide, and analysis by SMART program revealed that the protein possessed a single Ribosomal-S19 domain at the residues 45 to 130 (Fig 1A). Analysis by Antimicrobial Peptide Calculator and Predictor at APD revealed that the BjRPS15 had a total hydrophobic ratio of 36% and a net charge of +19, suggesting that BjRPS15 is a putative AMP. Prediction by CAMP showed that the amino acid residues 45-67 (RRFSRGLKRKHLALIKKLRKAKK, designated BjRPS15₄₅₋₆₇) were the core region of antimicrobial activity of BjRPS15 (Fig 1A). As shown in Table 1, the peptide BjRPS15₄₅₋₆₇ had a total hydrophobic ratio of 34% and a net charge of +12.04. The 3D modeling revealed that BjRPS15 was composed of 7 α -helices and 3 β -sheets (Fig 1B), and BjRPS15₄₅₋₆₇ comprised 2 α -helices (Fig 1C).

Protein sequence comparison showed that BjRPS15 shared 75.2% to

78.6% identity to the RPS15 of vertebrates, including mammals, reptiles, amphibians and fishes [3,31,32], and 79.6% to 81.6% identity to that of invertebrates [3] as well as 41.2% to 73.9% identity to prokaryotic homolog RPS19 [33] (S1 Fig). A search of the completed draft assembly and automated annotation of amphioxus genomes revealed the presence of a single cDNA and its genomic DNA sequence in both *B. belcheri* and *B. floridae* (transcript id: XM_019780268.1 for *B. belcheri* and XM_002594975.1 for *B. floridae*). Both the cDNAs of *B. belcheri* and *B. floridae* encoded a protein with 100% identity to BjRPS15, suggesting that in amphioxus RPS15 was absolutely conserved in interspecies. Analysis of the genomic structure uncovered that all the homologs of *BjRPS15* from different animals comprised 3 to 5 exons interspaced by 2 to 4 introns (Fig 1D), but each of their coding exons shared highly identical sequence, suggesting the general genomic sequence of *RPS15* retained rather stable throughout multicellular animal evolution.

Expression of *BjRPS15* after challenge with bacteria, LPS and LTA

qRT-PCR was used to examine the transcriptional profile of *BjRPS15* in the different tissues. As shown in Fig 2A, *BjRPS15* was predominantly expressed in the hepatic caecum, hind-gut, testis and ovary, and at a lower level in the gill, notochord and muscle, indicating that *BjRPS15* was expressed in a tissue-specific manner. Notably, the challenge with

the Gram-negative bacteria *A. hydrophila* and *E. coli* and the Gram-positive bacteria *S. aureus* and *B. subtilis* both resulted in significant increase in the expression of *BjRPS15* (Fig 2B). Similarly, the challenge with LPS and LTA also induced marked increase in the expression of *BjRPS15* (Fig 2C). These data suggested that BjRPS15 might be involved in the anti-infectious response in amphioxus.

Antibacterial activity of rBjRPS15 and BjRPS15₄₅₋₆₇

The purified recombinant proteins rBjRPS15 and rTRX both yielded a single band of approximately 21.85 and 20.4 kDa, respectively, well matching the expected sizes (Fig 3A). Western blotting showed that rBjRPS15 and rTRX were both reactive with the anti-His-tag antibody (Fig 3A), indicating that they were properly expressed. We then tested the antimicrobial activity of rBjRPS15 and rTRX (control) against the Gram-negative bacteria *A. hydrophila* and *E. coli* as well as the Gram-positive bacteria *S. aureus* and *B. subtilis*. As shown in Fig 3B, rBjRPS15 showed conspicuous antimicrobial activities against all the bacteria tested, with the minimum bactericidal concentration MBC (defined as the lowest concentration at which the bacterium was completely killed) against *A. hydrophila*, *E. coli* and *B. subtilis* being about 2 μ M and that against *S. aureus* being > 2 μ M. We also evaluated the MBC₅₀ (defined as the lowest concentration at which the 50%

bacterium was killed) of rBjRPS15 against *A. hydrophila*, *E. coli*, *S. aureus* and *B. subtilis*, which was all about 0.5 μ M. Similarly, BjRPS15₄₅₋₆₇ also showed bactericidal activities against *A. hydrophila*, *E. coli*, *S. aureus* and *B. subtilis*, with the MBC₅₀ against *A. hydrophila* and *E. coli* being about 2 μ M and that against *S. aureus* and *B. subtilis* about 4 μ M (Fig 3C). By contrast, rTRX showed little antimicrobial activity against all the bacteria tested (data not shown). These indicated that BjRPS15 was indeed an AMP with the residues 45-67 being the core region for the antimicrobial activity.

Antibacterial activity of BjRPS15₄₅₋₆₇ counterparts

To test if the antimicrobial activity of RPS15 was conserved during evolution, the counterparts of BjRPS15₄₅₋₆₇ ranging from prokaryotes to eukaryotes were investigated for the presence of antibacterial activity. First, sequence alignment showed that the sequence of BjRPS15₄₅₋₆₇ was highly conserved among the eukaryotes as well as the prokaryotes, with the residues of RPS19₃₃₋₅₅ of *Nitrospirae* sp. and RPS19₃₃₋₅₅ of *Aquificae* sp. being most divergent (Fig 4). Analysis by Antimicrobial Peptide Calculator and Predictor at APD revealed that *Nitrospirae* RPS19₃₃₋₅₅ had a hydrophobic ratio of 30% and a net charge of +5.08, *Aquificae* RPS19₃₃₋₅₅ a hydrophobic ratio of 21% and a net charge of +3.91, and all the other

188 BjRPS15₄₅₋₆₇ counterparts a hydrophobic ratio of >25% and a net
 189 charge of +10 to +12.5 (Table 1). The 3D modeling showed that all the
 190 3D structures of BjRPS15₄₅₋₆₇ counterparts,
 191 including *Nitrospirae* RPS19₃₃₋₅₅ and *Aquificae* RPS19₃₃₋₅₅, were similar
 192 to that of BjRPS15₄₅₋₆₇, consisting of 2 α -helices (S2 Fig). These suggested
 193 that the counterparts above might also have antibacterial activity. We thus
 194 synthesized the peptides of BjRPS15₄₅₋₆₇ counterparts, including *H.*
 195 *sapiens* RPS15₄₃₋₆₅, *X. tropicalis* RPS15₄₃₋₆₅, *D. rerio* RPS15₄₃₋₆₅, *A.*
 196 *planci* RPS15₄₆₋₆₈, *D. melanogaster* RPS15₄₆₋₆₈, *O. vulgaris* RPS15₅₀₋₇₂, *C.*
 197 *teleta* RPS15₄₉₋₇₁, *P. pacificus* RPS15₄₉₋₇₁, *S. pistillata* RPS15₄₄₋₆₆,
 198 *P. syringae* RPS19₅₀₋₇₂, *Nitrospirae* RPS19₃₃₋₅₅ and *Aquificae* RPS19₃₃₋₅₅,
 199 and examined their antibacterial activity. As shown in Table 2, all
 200 the peptides synthesized exhibited antimicrobial activities against *A.*
 201 *hydrophila* and *S. aureus*, that were basically comparable to or slightly
 202 lower than that of BjRPS15₄₅₋₆₇. All these data suggested that the
 203 emergence of the antibacterial activity of RPS15 could be traced to its
 204 prokaryotic homolog RPS19.

205 **Destruction of bacterial cells by rBjRPS15 and BjRPS15₄₅₋₆₇**

206 To examine the effects of rBjRPS15 and BjRPS15₄₅₋₆₇ on the morphology
 207 and structure of bacterial cells, both *A. hydrophila* and *S. aureus* were
 208 incubated with rBjRPS15 and BjRPS15₄₅₋₆₇, and subjected to

transmission electron microscopy examination. It was found that both rBjRPS15 and BjRPS15₄₅₋₆₇ caused a direct damage to the cells of *A. hydrophila* and *S. aureus*, resulting in membrane disruption and cytoplasmic leakage (Fig 5A and B). These indicated that rBjRPS15 and BjRPS15₄₅₋₆₇ were both bactericidal agents capable of directly killing the bacteria like *A. hydrophila* and *S. aureus*.

Bacterial and ligand-binding activities

We then tested if rBjRPS15 could interact with the bacteria. As revealed by Western blotting, rBjRPS15 had strong affinity to *A. hydrophila*, *E. coli*, *S. aureus* and *B. subtilis* (Fig 6A). By contrast, rTRX showed little affinity to the bacteria tested (Fig 6A). These indicated that rBjRPS15 could specifically interact with the Gram-negative and -positive bacteria.

The binding activity of rBjRPS15 to the ligands LPS and LTA was also detected. The results showed that rBjRPS15 was able to bind to LPS and LTA in a dose-dependent manner, whereas rTRX did not (Fig 6B). These indicated that rBjRPS15 interacted with the bacteria via LPS and LTA, suggesting that BjRPS15 might act as multivalent pattern recognition receptors.

Membrane depolarization by rBjRPS15 and BjRPS15₄₅₋₆₇

The membrane depolarization activities of rBjRPS15 and BjRPS15₄₅₋₆₇ were assayed using DiSC₃-5, a potential-dependent distributional

fluorescent dye. As shown in Fig 7, the fluorescence intensity of *A. hydrophila*, *E. coli*, *S. aureus* and *B. subtilis* cells treated with rBjRPS15 or BjRPS15₄₅₋₆₇ was all significantly increased, compared with control (treated with rTRX or PBS). This indicated that rBjRPS15 and BjRPS15₄₅₋₆₇ both caused depolarization of the bacterial plasma membrane.

Induction of intracellular ROS by rBjRPS15 and BjRPS15₄₅₋₆₇

High intracellular levels of ROS can cause apoptosis or necrosis. When *A. hydrophila*, *E. coli*, *S. aureus* or *B. subtilis* cells were treated with rBjRPS15 or BjRPS15₄₅₋₆₇, their intracellular ROS levels were significantly increased (Fig 8). These suggested that both rBjRPS15 and BjRPS15₄₅₋₆₇ might induce apoptosis/necrosis of the bacterial cells via increased production of intracellular ROS.

Non-toxicity of rBjRPS15 and BjRPS15₄₅₋₆₇ to mammalian cells

To test if rBjRPS15 and BjRPS15₄₅₋₆₇ were cytotoxic, their hemolytic activities towards human red blood cells (RBCs) were determined. As shown in Fig 9, neither rBjRPS15 nor BjRPS15₄₅₋₆₇ showed hemolytic activity towards human erythrocytes at all the concentrations tested. By contrast, RBCs incubated with 0.1% Triton X-100, which is usually used as full lysis control, exhibited remarkable hemolysis. The cytotoxicity of rBjRPS15 and BjRPS15₄₅₋₆₇ to murine RAW264.7 cells was also

examined by measuring the cell viability via MTT method. As shown in Table 3, rBjRPS15 and BjRPS15₄₅₋₆₇ were neither toxic to murine RAW264.7 cells at the concentrations tested. These showed that neither rBjRPS15 nor BjRPS15₄₅₋₆₇ were toxic to mammalian cells, suggesting that they both showed a high bacterial membrane selectivity.

Presence of extracellular RPS15 in vivo

Next, we examined if extracellular RPS15 was present in animals. Western blotting revealed that the amphioxus humoral fluid was reactive with anti-RPS15 monoclonal antibody, yielding a single band of ~17 kDa (Fig 10A), well matching the molecular mass predicted by BjRPS15 gene, suggesting the presence of extracellular RPS15 in amphioxus. This was clearly supported by LC/MS/MS analysis (Fig 10B). Similarly, RPS15 was also found to be present in the shrimp hemolymph as well as in zebrafish and mouse sera (Fig 10A). All these indicated that RPS15 existed as extracellular form across widely different animals ranging from invertebrate species to mammals.

Discussion

Proteins that fulfil two or more distinct and physiologically relevant biochemical or biophysical functions independent of gene fusions or multiple RNA splice variants are called moonlighting proteins [34,35]. It is well-known that ribosomal protein S15, or RPS15, a component of the

272 40S subunit, functions in protein synthesis [36]. In this study, we show
 273 for the first time that amphioxus RPS15, BjRPS15, is a previously
 274 uncharacterized AMP. It not only functions as a multiple pattern
 275 recognition receptor, capable of recognizing LPS and LTA, but also as a
 276 bactericidal effector, capable of killing a wide spectrum of bacteria.
 277 Potential modes of action of AMPs include interacting with or inserting
 278 into bacterial membrane, which can cause lethal depolarization of the
 279 usually polarized membrane, scrambling of the normal distribution of
 280 lipids between the leaflets of the bilayer, formation of physical pores and
 281 loss of critical intracellular targets. We clearly demonstrate that BjRPS15
 282 executes function by a combined action of membranolytic mechanisms
 283 including interaction with bacterial membrane through LPS and LTA as
 284 well as membrane depolarization. BjRPS15 can also stimulate production
 285 of intracellular ROS in bacteria, which may lead to apoptosis/necrosis of
 286 the bacterial cells. These indicate that BjRPS15 is a novel member of
 287 moonlighting protein, which, in addition to participation in protein
 288 synthesis, is involved in anti-infectious response in amphioxus.
 289 Intriguingly, RPS15 is present in the humoral fluid of amphioxus,
 290 hemolymph of shrimp and sera of zebrafish and mice, indicating that it
 291 exists across widely separated taxa. This also has a physiological
 292 implication that RPS15 may extracellularly play a critical role in
 293 systematic immunity in different animals, protecting them against

294 bacterial infection by interacting with and destructing potential
295 pathogens.

296 Looking at the amino acid sequences, BjRPS15 shares significantly
297 high identity to its eukaryotic as well as prokaryotic homologs. The 3D
298 structures of BjRPS15 homologs are very similar among both eukaryotes
299 and prokaryotes. Furthermore, the antibacterial features such as high
300 hydrophobic ratio and net positive charge clearly exist in RPS19, an early
301 RPS15 orthologue of bacteria, including *Nitrospirae* sp.,
302 *Aquificae* sp. and *P. syringae*. Importantly, it was experimentally proven
303 that the synthesized the peptides of BjRPS15₄₅₋₆₇ counterparts, including
304 *H. sapiens* RPS15₄₃₋₆₅, *X. tropicalis* RPS15₄₃₋₆₅, *D. rerio* RPS15₄₃₋₆₅, *A.*
305 *planci* RPS15₄₆₋₆₈, *D. melanogaster* RPS15₄₆₋₆₈, *O. vulgaris* RPS15₅₀₋₇₂, *C.*
306 *teleta* RPS15₄₉₋₇₁, *P. pacificus* RPS15₄₉₋₇₁, *S. pistillata* RPS15₄₄₋₆₆,
307 *P. syringae* RPS19₅₀₋₇₂, *Nitrospirae* RPS19₃₃₋₅₅ and *Aquificae* RPS19₃₃₋₅₅,
308 all show conspicuous antibacterial activities against the Gram-negative
309 and -positive bacteria tested. These suggest that the antibacterial
310 properties of this family of molecules are very ancient and highly
311 conserved.

312 An important point about the exploitation of membranolytic
313 antimicrobial therapeutics is that they cannot be cytotoxic to mammalian
314 cell membrane. We find that both BjRPS15 and its truncated

BjRPS15₄₅₋₆₇ are virtually not toxic towards mammalian cells including human erythrocytes and murine macrophages RAW264.7. This denotes that they possess high membrane selectivity to bacterial cells but not to mammalian cells, rendering them ideal lead molecules for the exploitation of new peptide antibiotics against bacteria.

In summary, this present study highlights that RPS15 is a new AMP functioning as a multiple sensor, capable of recognizing LPS and LTA, and an effector, capable of killing the potential pathogens. It also suggests that the antibacterial activities of this family of molecules have ancient origin and high conservation.

Materials and methods

Animal culture

All animal experiments performed here conformed to the ethical guidelines established by the Institutional Animal Care and Use Committee of the Ocean University of China. Adult amphioxus (*Branchiostoma japonicum*) collected during the breeding season in the vicinity of Qingdao, China was cultured in aerated seawater at room temperature, and fed twice a day with single-celled algae. Shrimps (*Fenneropenaeus chinensis*) with body weight of approximately 10 to 20 g were purchased from Xiaogang market in Qingdao, and cultured in aerated seawater at room temperature. Wild-type zebrafish (*Danio rerio*)

aged 2-3 months purchased from a local fish dealer were maintained at 27 ± 1°C under a controlled light cycle (14 h light/10 h dark) in a 20 L tank with well-aerated tap water, and fed on live bloodworms and Miero Fish Food twice a day. Mice (*Mus musculus*) aged 11 months (no. 20140007) were from Jinan Pengyue Laboratory Animal Breeding Co., Ltd, and housed one per cage in an environmentally controlled atmosphere (temperature 22°C and relative humidity 56%) with a 12 h light/dark cycle. They were given free access to water and diet and provided with shredded wood floor bedding. All the animals were acclimatized for one week before the experiments.

RNA extraction and cDNAs synthesis

Total RNAs were extracted with Trizol (TaKaRa, China) from *B. japonicum* according to the manufacturer's instructions. After digestion with the recombinant RNase-free DNase (TaKaRa) to eliminate the genomic contamination, cDNAs were synthesized with reverse transcription kit (TaKaRa) with oligo d(T) primer. The reaction was carried out at 42°C for 50 min and inactivated at 75°C for 15 min. The cDNAs synthesized were stored at -20°C until use.

Cloning and sequencing of *BjRPS15*

Based on the sequence of *B. belcheri* RPS15 gene (accession number: XP_019635827) in the database of NCBI (<http://www.ncbi.nlm.nih.gov/>),

a pair of primers P1 and P2 (S1 Table) was designed using Primer Premier 5.0 program. The PCR amplification reaction was carried out at 94°C for 5 min, followed by 32 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 7 min. The amplification products were gel-purified using DNA gel extraction kit (AXYGEN), cloned into the pGEM-T vector (Invitrogen), and transformed into Trans5a *Escherichia coli* (TransGen). The positive clones were selected and sequenced to verify for authenticity.

Sequence analysis

The domains and signal peptide of the deduced protein were analyzed by the SMART program (<http://smart.embl-heidelberg.de/>) and SignalP 5.0 Server (<http://www.cbs.dtu.dk/services/SignalP/>), respectively. The molecular weight (MW) and isoelectric points (pI) of the protein were determined by the ProtParam (<http://www.expasy.ch/tools/protparam.html>). Homology searches in the GenBank database were carried out by BLAST server (<http://www.ncbi.nlm.nih.gov/BLAST/>). The information of exon-intron organization was obtained from NCBI database. Multiple protein sequences were aligned using the MegAlign program of the LASERGENE software suite (DNASTAR). The SWISS-MODEL prediction algorithm (<https://swissmodel.expasy.org/>) was applied to

generate the three-dimensional (3D) structure model. CAMP server (<http://www.camp.bicnirrh.res.in/predict/>) was used to predict the core sites for antimicrobial activity, and Antimicrobial Peptide Calculator and Predictor at APD (<http://aps.unmc.edu/AP/main.php>) used to calculate the total hydrophobic ratio and net charge.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was used to examine the transcriptional profile of *BjRPS15* in the different tissues of *B. japonicum*, including the hepatic caecum, hind-gut, gill, muscle, notochord, testis and ovary, as described by Wang et al. [14] and Yang et al. [15]. The PCR primer pairs P3 and P4 as well as P5 and P6 (S1 Table) specific of *BjRPS15* and *EF1α* were designed using primer 5.0 program. The *EF1α* gene was chosen as the reference for internal standardization. The expression level of *BjRPS15* relative to that of *EF1α* gene was calculated by the comparative Ct method ($2^{-\Delta\Delta Ct}$) [16].

The qRT-PCR was also performed to assay the transcriptional profile of *BjRPS15* in response to challenge with the bacteria *Aeromonas hydrophila* (ATCC 35654), *E. coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis* (ATCC 6633) as well as the bacterial signature molecules lipopolysaccharide (LPS) and lipoteichoic acid (LTA) as described by Wang et al. [14]. The *B. japonicum* were

cultured in 1 L of sterilized seawater containing 10^8 cells/ml of *A. hydrophila*, *E. coli*, *S. aureus* or *B. subtilis* or 10 µg/ml of the bacterial signature molecules LPS (Sigma, USA) or LTA (Sigma, USA) [14,17,18], and sampled at 0, 2, 4, 8, 12, 24, 48, and 72 h after the exposure. Extraction of total RNAs, cDNA synthesis and qRT-PCR were carried out as above.

Construction of expression vector

The sequence encoding BjRPS15 was amplified by PCR using the primer pairs P7 and P8 (S1 Table) with *EcoR* I and *Xho* I sites in the forward and reverse primers, respectively. The PCR products were sub-cloned into the plasmid expression vector pET-28a (Novagen) previously cut with the restriction enzymes *EcoR* I and *Xho* I. The identity of inserts was verified by sequencing, and the constructed plasmid was designated *pET-28a/BjRPS15*.

Expression and purification of rBjRPS15

The plasmid *pET28a/BjRPS15* was transformed into *E. coli* transetta (DE3) cells. Induced expression and purification of recombinant BjRPS15, rBjRPS15, were performed as described by Gao et al. [19]. Recombinant thioredoxin His Tag (rTRX) used for control was similarly prepared. The purity of the eluted samples and purified proteins were analyzed by a 12% SDS-PAGE gel and stained with Coomassie brilliant

blue R-250. The concentrations of the recombinant proteins were determined by BCA method.

Western blotting

Western blotting was conducted as described by Yao et al. [20]. The anti-His-tag mouse monoclonal antibody (CWBIO) used was diluted 1:4000 with 4% BSA in PBS.

Antimicrobial activity assay

The antimicrobial activity of rBjRPS15 was assayed by the method of Shi et al. [21]. Briefly, aliquots of 50 μ l of *A. hydrophila*, *E. coli*, *S. aureus* or *B. subtilis* suspension (3×10^4 cells/ml) were each mixed with 50 μ l of rBjRPS15 solution (0, 0.5, 1, 2, 3, and 4 μ M) and incubated at 25°C for 1 h. Each of the bacterial mixtures was then plated onto 3 agar plates (30 μ l/plate). After incubation at 37°C for 12 h, the resulting bacterial colonies in each plate were counted. The percent of bactericidal activity was calculated as follows: [number of colonies (control-test)/number of colonies (control)] $\times$ 100 (n = 3). The rTRX was used as control.

The core site of BjRPS15 for antimicrobial activity was predicted by CAMP server, and the residues positioned at 45-67 (RRFSRGLKRKHLALIKKLRKAKK), designated BjRPS15₄₅₋₆₇, were identified as the only candidate. Therefore, BjRPS15₄₅₋₆₇ was synthesized by Shanghai Sangon Biological Engineering Technology & Services Co.,

Ltd, using standard solid-phase Fmoc method, and used for antimicrobial activity assay. Because C-terminal amidation is common in antimicrobial peptides (AMPs) [22], the C-termini of the peptide was thus amidated. The peptide synthesized was purified to >95% by high-performance liquid chromatography (HPLC) and the mass of the synthetic peptide was verified by mass spectrometer (lcms-2010a, Shimadzu, Japan). The peptide was dissolved in PBS (2 mg/ml) and stored at -80°C till used. The antimicrobial activity of BjRPS15₄₅₋₆₇ (0, 0.625, 1.25, 2.5, 5, 10 µM) was determined as described above.

Sequence alignment revealed that BjRPS15 shared more than 59.7% identity to eukaryotic RPS15 and more than 41.2% identity to its prokaryotic homolog RPS19 (S1 Fig). Thus, we synthesized the core regions (corresponding to the region BjRPS15₄₅₋₆₇) of RPS15 of the eukaryotes including *Homo sapiens*, *Xenopus tropicalis*, *Danio rerio*, *Acanthaster planci*, *Drosophila melanogaster*, *Octopus vulgaris*, *Capitella teleta*, *Pristionchus pacificus* and *Stylophora pistillata* as well as those of RPS19 of the prokaryotes including *Pseudomonas syringae*, *Nitrospirae* sp. and *Aquificae* sp. (see Fig 4 below) to test the conservation of RPS15 antibacterial activity. The antibacterial activities of the synthesized peptides were examined as above.

Transmission electron microscopy (TEM)

TEM was performed to test the effect of rBjRPS15 and BjRPS15₄₅₋₆₇ on the morphology and structures of the bacteria *A. hydrophila* and *S. aureus* as described by Liu et al. [23]. Briefly, aliquots of 500 µl of the bacterial suspensions containing 5×10^7 cells/ml were mixed with 500 µl of rBjRPS15 (4 µM) or BjRPS15₄₅₋₆₇ (20 µM), respectively. In parallel, aliquots of 500 µl of the bacterial suspensions were mixed with 500 µl PBS as control. The mixtures were incubated at 25°C for 1 h, fixed in 2.5% glutaraldehyde in 100 mM PBS, and then dropped onto 400-mesh carbon-coated grids and allowed to stand at room temperature for 3 min for negative staining. Excess fluid was removed by touching the edge of filter paper. The grids were then put into 2% phosphotungstic acid for 3 min, dried by filter paper, and observed under a JEOL JSM-840 transmission electron microscope.

Bacterial binding assay

To test the bacterial binding activity of rBjRPS15, the bacteria *A. hydrophila*, *E. coli*, *S. aureus* and *B. subtilis* were cultured to mid-logarithmic phase, and collected by centrifugation at 6000 g for 5 min. After washing twice with PBS, the bacteria were re-suspended in PBS, giving a density of 1×10^8 cells/ml. Aliquots of 300 µl of bacterial suspensions were mixed with 150 µl of 1 µM rBjRPS15 or rTRX (control), respectively. The mixtures were incubated at 25°C for 1 h, and

centrifuged at 6000 g at 4°C for 5 min. The bacterial pellets were washed three times with PBS and re-suspended in 300 µl PBS. The bacterial suspensions were subjected to 12% SDS-PAGE gel and the binding activity was determined by Western blotting as described above.

Ligand binding assay

An enzyme-linked immunosorbent assay (ELISA) was preformed to examine the binding of rBjRPS15 to the ligands LPS and LTA. Aliquots of 50 µl of 40 µg/ml LPS or LTA were applied to each well of a 96-well microplate and air-dried at 25°C overnight. The plates were incubated at 60°C for 30 min to fix the ligands, and then each well was blocked with 100 µl of 1 mg/ml BSA in PBS at 37°C for 2 h. After washing four times with PBST, a total of 100 µl PBS containing 0.1 mg/ml BSA and different concentrations (0, 0.0625, 0.125, 0.25, 0.5, 1, 1.5 and 2 µM) of rBjRPS15 or rTRX (control) was added into each well and incubated at 25°C for 3 h. The wells were rinsed four times with PBST, and incubated with 100 µl of mouse anti-His-tag antibody (CWBIO), diluted 1:5000 with 4% BSA in PBS, at 37°C for 1 h. After washing four times with PBST, the wells was then incubated with 100 µl of HRP-labeled goat anti-mouse IgG Ab (CWBIO), diluted 1:8000 with 4% BSA in PBS, at room temperature for 1 h. Subsequently, the wells were washed four times with PBST, added with 75 µl of 0.4 mg/ml O-phenylenediamine

(Amresco) in the buffer consisting of 51.4 mM Na₂HPO₄, 24.3 mM citric acid and 0.045% H₂O₂ (pH5.0), and reacted at 37°C for 10 min. Finally, 25 µl of 2 M H₂SO₄ was added into each well to terminate the reaction, and absorbance at 492 nm was monitored by a microplate reader (GENios Plus; Tecan).

Membrane depolarization assay

The assay for membrane depolarization activity of rBjRPS15 and BjRPS15₄₅₋₆₇ was performed with the membrane potential-sensitive dye 3,3'-dipropylthiacarbocyanine iodide (DiSC₃-5; Sigma) and the bacteria *A. hydrophila*, *E. coli*, *S. aureus* and *B. subtilis*, according to the method of Lee et al. [24]. The bacterial cells in the mid-logarithmic phase were harvested by centrifugation at 6000 g for 10 min, washed in 5 mM HEPES buffer (pH7.3) containing 20 mM glucose, and re-suspended in 5 mM HEPES buffer containing 20 mM glucose and 100 mM KCl to an OD₆₀₀ of 0.05. Aliquots of 100 µl of the bacterial suspensions, supplemented with 0.5 µM DiSC₃-5, were applied to each well of a 96-well flat bottom white microplate, and allowed to stand for 30 min at room temperature to get a steady baseline of fluorescence intensity. The bacterial suspensions were then mixed with 100 µl of PBS containing 2 µM rBjRPS15, 2 µM rTRX, 10 µM BjRPS15₄₅₋₆₇ or PBS alone. rTRX and PBS were used as control. Changes in the fluorescence intensity were

continuously recorded for 30 min with a TECAN-GENios plus spectrofluorimeter at an excitation wavelength of 622 nm and an emission wavelength of 670 nm.

Reactive oxygen species assay

The levels of reactive oxygen species (ROS) were measured as described by Cui et al. [25]. The bacteria *A. hydrophila*, *E. coli*, *S. aureus* and *B. subtilis* were re-suspended in the corresponding culture medium containing 10 μ M DCFH₂-DA, yielding a density of 10⁷ cells/ml. After incubation at 37°C for 30 min, the bacteria were collected by centrifugation at 6000 g at room temperature for 10 min. The bacterial cells were washed three times with PBS, re-suspended in 1 ml of PBS containing 1 μ M rBjRPS15, 1 μ M rTRX or 5 μ M BjRPS15₄₅₋₆₇. For positive control, the bacterial cells were re-suspended in 1 ml PBS containing 50 μ g/ml Rosup, a compound mixture, that can significantly increase ROS levels in cells within 30 min. For blank control, the cells were re-suspended in 1 ml PBS alone. The bacterial suspensions were incubated at 25°C for 1 h and the fluorescence intensities were recorded immediately with a TECAN-GENios plus spectrofluorimeter at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Hemolytic activity assay

Human red blood cells (RBCs) were used to test the hemolytic activity of

rBjRPS15 and BjRPS15₄₅₋₆₇ as described by Hu et al. [26]. Healthy human blood was obtained and placed in an EDTA anticoagulant tube. The RBCs were collected by centrifugation at room temperature at 1000 g for 10 min. After washing three times with PBS, the RBC pellets were suspended in PBS to give a concentration of 4% (v/v). Aliquots of 200 µl RBCs suspension were mixed with 200 µl of different concentrations of rBjRPS15 (0, 1.25, 2.5, 5 and 10 µM) or BjRPS15₄₅₋₆₇ (0, 1.25, 2.5, 5 and 10 µM), respectively. After incubation at 37°C for 1 h, the mixtures were centrifuged at room temperature at 1000 g for 10 min. The supernatants were collected and added into a 96-well plate. The absorbance was measured at 540 nm under a microplate reader (Multiskan GO; Thermo Scientific). RBCs incubated with PBS, rTRX solution (10 µM), and 0.1% Triton X-100 solution served as blank, negative and positive controls, respectively.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

To test if rBjRPS15 and BjRPS15₄₅₋₆₇ are cytotoxic to murine RAW264.7 cells, MTT assay was performed as described by Hu et al. [26]. RAW264.7 cells were suspended in serum-free DMEM and aliquots of 180 µl of the cell suspension (1×10^6 cells/ml) were sampled into a 96-well plate and cultured at 37°C with 5% CO₂ for 2 h. After removal of

the medium, aliquots of 200 μ l serum-free DMEM with different concentrations of rBjRPS15 (0, 1.25, 2.5, 5 and 10 μ M) and BjRPS15₄₅₋₆₇ (0, 1.25, 2.5, 5 and 10 μ M) were added to each well of a 96-well microplate, incubated for 4 h, and then 20 μ l of MTT solution (5 mg/ml) was added into each well. After incubation for another 4 h, the medium was removed and 150 μ l of dimethyl sulfoxide (DMSO) was added. The absorbance at 492 nm was measured under a microplate reader. For control, the solution of rBjRPS15 and BjRPS15₄₅₋₆₇ was substituted by PBS alone, and the assays were similarly processed. The percent viability against the control was calculated as follows: (OD of treated groups/OD of control groups) $\times$ 100 (n = 3).

Assay for extracellular RPS15 in vivo

Western blotting was used to test if RPS15 was present in the humoral fluid of amphioxus, hemolymph of shrimp and sera of zebrafish and mouse. The humoral fluid was prepared by the method of Pang et al. [27], the hemolymph prepared from shrimps by the method of Zhang et al. [28], and the sera of zebrafish and mouse were prepared as described by Babaei et al. [29] and Greenfield [30]. Western blotting was performed as described above using anti-RPS15 monoclonal antibody (1:1000; Abcam, UK) and anti-GAPDH antibody (1:3000; Bioss, China) as the primary antibodies, respectively, and HRP-labeled antibody

(1:6000; CWBIO, China) as the secondary antibody. The bands were visualized by ECL Western blotting substrate (Thermo Fisher Scientific, USA).

To further verify the presence of BjRPS15 in the humoral fluid of amphioxus, the liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis was performed. The humoral fluid was run on a 12% SDS-PAGE gel and stained with Coomassie Brilliant Blue R-250. The band with an expected molecular weight of ~17 kDa corresponding that of RPS15 was cut off and subjected to LC/MS/MS analysis (Beijing Protein Innovation Co., Ltd, China).

Statistical analysis

The experiments were performed in triplicate, and repeated three times. Data were subjected to statistical evaluation with unpaired t-test, and the value $p < 0.05$ was considered as significant. All the data were expressed as mean $\pm$ SEM.

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References

1. Wang T, Hou Y, Ding X, Song B, Wang F, et al. (2013) Overexpression, purification, molecular characterization and pharmacological evaluation for anticancer activity of ribosomal protein S23 from the giant panda (*Ailuropoda melanoleuca*). Mol Med Rep 7: 1875-1882. pmid: 23591681
2. Wool IG, Chan YL, Glück A (1995) Structure and evolution of mammalian ribosomal proteins. Biochem Cell Biol 73: 933-947. pmid: 8722009
3. Khairulina J, Graifer D, Bulygin K, Ven'yaminova A, Frolova L, et al. (2010) Eukaryote-specific motif of ribosomal protein S15 neighbors A site codon during elongation and termination of translation. Biochimie 92: 820-825. pmid: 20206660
4. Martin I, Kim JW, Lee BD, Kang HC, Xu JC, et al. (2014) Ribosomal protein s15 phosphorylation mediates LRRK2 neurodegeneration in Parkinson's disease. Cell 157: 472-485. pmid: 24725412
5. Zhou X, Liao WJ, Liao JM, Liao P, Lu H (2015) Ribosomal proteins: functions beyond the ribosome. J Mol Cell Biol 7:

- 629 92-104. pmid: 25735597
- 630 6. Hultmark D, Engström A, Bennich H, Kapur R, Boman HG
631 (1982) Insect immunity: isolation and structure of cecropin D
632 and four minor antibacterial components from *Cecropia* pupae.
633 Eur J Biochem 127: 207-217. pmid: 7140755
- 634 7. Hultmark D, Steiner H, Rasmuson T, Boman HG (1980) Insect
635 immunity. Purification and properties of three inducible
636 bactericidal proteins from hemolymph of immunized pupae of
637 *Hyalophora cecropia*. Eur J Biochem 106: 7-16. pmid:
638 7341234
- 639 8. Steiner H, Hultmark D, Engström A, Bennich H, Boman HG
640 (1981) Sequence and specificity of two antibacterial proteins
641 involved in insect immunity. Nature 292: 246-248. pmid:
642 7019715
- 643 9. Pütsep K, Brändén CI, Boman HG, Normark S (1999)
644 Antibacterial peptide from *H. pylori*. Nature 398: 671-672.
645 pmid: 10227288
- 646 10. Pütsep K, Normark S, Boman HG (1999) The origin of
647 cecropins; implications from synthetic peptides derived from
648 ribosomal protein L1. FEBS Lett 451: 249-252. pmid:
649 10371199

11. Pidutti P, Federici F, Brandi J, Manna L, Rizzi E, et al. (2017)
Purification and characterization of ribosomal proteins L27 and
L30 having antimicrobial activity produced by the
Lactobacillus salivarius SGL03. J Appl Microbiol 124:
398-407. pmid: 29159916
12. Seo JK, Kim DG, Oh R, Park KS, Lee IA, et al. (2017)
Antimicrobial effect of the 60S ribosomal protein L29
(cgRPL29), purified from the gill of pacific oyster, *Crassostrea*
gigas. Fish Shellfish Immunol 67: 675-683. pmid: 28663127
13. Fernandes JM, Smith VJ (2002) A novel antimicrobial function
for a ribosomal peptide from rainbow trout skin. Biochem
Biophys Res Commun 296: 167-171. pmid: 12147245
14. Wang G, Zhang S, Wang Z (2009) Responses of alternative
complement expression to challenge with different
combinations of *Vibrio anguillarum*, *Escherichia coli* and
Staphylococcus aureus: evidence for specific immune priming
in amphioxus *Branchiostoma belcheri*. Fish Shellfish Immunol
26: 33-39. pmid: 18983925
15. Yang G, Guo H, Li H, Shan S, Zhang X, et al. (2014) Molecular
characterization of LEAP-2 cDNA in common carp (*Cyprinus*
carpio L.) and the differential expression upon a *Vibrio*

- 671 anguillarum stimulus; indications for a significant immune role
672 in skin. Fish Shellfish Immunol 37: 22-29. pmid: 24418455
- 673 16. Livak KJ, Schmittgen TD (2001) Analysis of Relative Gene
674 Expression Data Using Real-Time Quantitative PCR and the
675 $2^{-\Delta\Delta C_t}$ Method. Methods 25: 402-408. pmid: 11846609
- 676 17. Fan C, Zhang S, Li L, Chao Y (2008) Fibrinogen-related protein
677 from amphioxus *Branchiostoma belcheri* is a multivalent
678 pattern recognition receptor with a bacteriolytic activity. Mol
679 Immunol 45: 3338-3346. pmid: 18533266
- 680 18. Wang Y, Zhang S (2011) Identification and expression of
681 liver-specific genes after LPS challenge in amphioxus: the
682 hepatic cecum as liver-like organ and “pre-hepatic” acute phase
683 response. Funct Integr Genomics 11: 111-118. pmid: 21052758
- 684 19. Gao Z, Qu B, Yao L, Ma Z, Cui P, et al. (2018) Identification
685 and functional characterization of amphioxus Miple, ancestral
686 type of vertebrate midkine/pleiotrophin homologues. Dev
687 Comp Immunol 89: 31-43. pmid: 30096337
- 688 20. Yao L, Qu B, Ma Z, Chen Y, Tan Y, et al. (2019) Lectin-like and
689 bacterial-agglutinating activities of heat shock proteins Hsp5
690 and Hsp90 α from amphioxus *Branchiostoma japonicum*. Fish
691 Shellfish Immunol 95: 688-696. pmid: 31683002

21. Shi X, Zhang S, Pang Q (2006) Vitellogenin is a novel player in defense reactions. *Fish Shellfish Immunol* 20: 769-772. pmid: 16293421
22. Lee IH, Zhao C, Cho Y, Harwig SS, Cooper EL, et al. (1997) Clavanins, α -helical antimicrobial peptides from tunicate hemocytes. *FEBS Lett* 400: 158-162. pmid: 9001389
23. Liu H, Lei M, Du X, Cui P, Zhang S (2015) Identification of a novel antimicrobial peptide from amphioxus *Branchiostoma japonicum* by in *silico* and functional analyses. *Sci Rep* 5: 18355. pmid: 26680226
24. Lee H, Hwang JS, Lee J, Kim JI, Lee DG (2015) Scolopendin 2, a cationic antimicrobial peptide from centipede, and its membrane-active mechanism. *Biochim Biophys Acta* 1848: 634-642. pmid: 25462167
25. Cui P, Dong Y, Li Z, Zhang Y, Zhang S (2016) Identification and functional characterization of an uncharacterized antimicrobial peptide from a ciliate *Paramecium caudatum*. *Dev Comp Immunol* 60: 53-65. pmid: 26883426
26. Hu L, Sun C, Wang S, Su F, Zhang S (2013) Lipopolysaccharide neutralization by a novel peptide derived from phosvitin. *Int J Biochem Cell Biol* 45: 2622-2631. pmid: 24028820

- 713 27. Pang Q, Zhang S, Wang C, Shi X, Sun Y (2004) Presence of
714 prophenoloxidase in the humoral fluid of
715 amphioxus *Branchiostoma belcheri tsingtauense*. Fish Shellfish
716 Immunol 17:477-487. pmid: 15313513
- 717 28. Zhang XW, Xu WT, Wang XW, Mu Y, Zhao XF, et al. (2009) A
718 novel C-type lectin with two CRD domains from Chinese
719 shrimp *Fenneropenaeus chinensis* functions as a pattern
720 recognition protein. Mol Immunol 46: 1626-1637. pmid:
721 19328552
- 722 29. Babaei F, Ramalingam R, Tavendale A, Liang Y, Yan LS, et al.
723 (2013) Novel blood collection method allows plasma proteome
724 analysis from single zebrafish. J Proteome Res 12: 1580-1590.
725 pmid: 23413775
- 726 30. Greenfield EA (2017) Sampling and Preparation of Mouse and
727 Rat Serum. Cold Spring Harb Protoc 11: 903-908. pmid:
728 29093207
- 729 31. Takasawa S, Tohgo A, Unno M, Shiga K, Yonekura H, et al.
730 (1992) The primary structure of rat rig/ribosomal protein S15
731 gene. Biochim Biophys Acta 1132: 228-230. pmid: 1390896
- 732 32. Uechi T, Nakajima Y, Nakao A, Torihara H, Chakraborty A, et al.
733 (2006) Ribosomal Protein Gene Knockdown Causes

734 Developmental Defects in Zebrafish. Plos one 1: e37. pmid:
735 17183665

736 33. Butterfield CN, Li Z, Andeer PF, Spaulding S, Thomas BC, et al.
737 (2016) Proteogenomic analyses indicate bacterial methylotrophy
738 and archaeal heterotrophy are prevalent below the grass root zone.
739 Peer J 4: e2687. pmid: 27843720

740 34. Jeffery CJ (1999) Moonlighting proteins. Trends Biochem Sci
741 24: 8-11. pmid: 10087914

742 35. Jeffery CJ (2017) Protein moonlighting: what is it, and why is it
743 important? Philos Trans R Soc Lond B Biol Sci 373: 1738.
744 pmid: 29203708

745 36. Hou WR, Luo XY, Du YJ, Tian MJ (2008) cDNA cloning and
746 sequences analysis of *RPS15* from the Giant Panda. Recent Pat
747 DNA Gene Seq 2: 16-19. pmid: 19075940

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763 **Table 1.** Amino acid sequences and chemical properties of BjRPS15 related peptides.

764 pI, isoelectric point. MW, molecular weight (kDa).

Name	Sequence	Hydrophobic ratio	Net charge	pI	MW
<i>Homo sapiens</i> RPS15 ₄₃₋₆₅	RRLNRGLRRKQHSLLKRLRKAKK	26%	+12.07	12.88	2.912
<i>Xenopus tropicalis</i> RPS15 ₄₃₋₆₅	RRLNRGLRRKQNSLLKRLRKAKK	26%	+11.90	12.88	2.889
<i>Danio rerio</i> RPS15 ₄₃₋₆₅	RRLNRGLRRKQQSLLKRLRKAKK	26%	+11.90	12.88	2.903
BjRPS15 ₄₅₋₆₇	RRFSRGLKRKHLALIKKLRKAKK	34%	+12.07	12.71	2.832

<i>Acanthaster planci</i>	RKFNRGLKRKPLALLKKLRKAKK	34%	+11.90	12.59	2.791
RPS15 ₄₆₋₆₈					
<i>Drosophila melanogaster</i>	RRFSRGLKRKPMALIKKLRKAKK	34%	+11.90	12.71	2.810
RPS15 ₄₆₋₆₈					
<i>Octopus vulgaris</i>	RRFTRGLKRKPMALIKRLRKAKK	34%	+11.90	12.80	2.852
RPS15 ₅₀₋₇₂					
<i>Capitella teleta</i>	RRMTRGLKRKPMALIKRLRKAKK	34%	+11.90	12.80	2.836
RPS15 ₄₉₋₇₁					
<i>Pristionchus pacificus</i>	RRLSRGLKRKHLALLARLQKAKK	39%	+10.07	12.71	2.740
RPS15 ₄₉₋₇₁					
<i>Stylophora pistillata</i>					
RPS15 ₄₄₋₆₆					
<i>Pseudomonas syringae</i>	RRFSRGLKRKPVHLMKRLRKAKK	30%	+12.07	12.80	2.890
RPS19 ₅₀₋₇₂					
Nitrospirae bacterium	RRINRGLKRKPMGLIKKLRKAKQ	30%	+10.90	12.71	2.788
RPS19 ₃₃₋₅₅					
Aquificae bacterium	RSLVKGLTNDQRTLMEHVRRAR	30%	+5.08	11.93	2.764
RPS19 ₃₃₋₅₅	K				
		21%	+3.91	10.93	2.710
	RSLRRGLTDEQRKVLEKLRKGDG				

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767 **Table 2.** Minimum bactericidal concentration (MBC) and 50% minimum bactericidal
768 concentration (MBC₅₀) of BjRPS15 related peptides against the bacteria.

Name	MBC (μM)		MBC ₅₀ (μM)	
	<i>A. hydrophila</i>	<i>S. aureus</i>	<i>A. hydrophila</i>	<i>S. aureus</i>
<i>Homo sapiens</i> RPS15 ₄₃₋₆₅	10	10	2	2
<i>Xenopus tropicalis</i> RPS15 ₄₃₋₆₅	10	10	2	2
<i>Danio rerio</i> RPS15 ₄₃₋₆₅	10	10	2	2
BjRPS15 ₄₅₋₆₇	>10	>10	2	4
<i>Acanthaster planci</i> RPS15 ₄₆₋₆₈	>10	>10	2	4
<i>Drosophila melanogaster</i> RPS15 ₄₆₋₆₈	>10	>10	2	4
<i>Octopus vulgaris</i> RPS15 ₅₀₋₇₂	>10	>10	2	4
<i>Capitella teleta</i> RPS15 ₄₉₋₇₁	>10	>10	2	4
<i>Pristionchus pacificus</i> RPS15 ₄₉₋₇₁	>10	>10	2	4
<i>Stylophora pistillata</i> RPS15 ₄₄₋₆₆	>10	>10	2	4
<i>Pseudomonas syringae</i> RPS19 ₅₀₋₇₂	>10	>10	2	4
Nitrospirae bacterium RPS19 ₃₃₋₅₅	>10	>10	10	>10

Aquificae bacterium RPS19₃₃₋₅₅ >10 >10 10 >10

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773 **Table 3.** The percent viability of RAW264.7 cells in the presence of rBjRPS15 and

774 BjRPS15₄₅₋₆₇. Data were expressed as mean $\pm$ SEM (n = 3).

	rBjRPS15		BjRPS15 ₄₅₋₆₇	
	Concentration (μ M)	Viability (%)	Concentration (μ M)	Viability (%)
	0	100	0	100
	1.25	102 $\pm$ 2	1.25	106 $\pm$ 4
	2.5	103 $\pm$ 3	2.5	107 $\pm$ 3
	5	102 $\pm$ 4	5	110 $\pm$ 3
	10	105 $\pm$ 3	10	106 $\pm$ 5

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795 **Figure legends**

796 **Fig 1.** The full-length nucleotide and deduced amino acid sequences, 3D
 797 molecular modeling and genomic organization of BjRPS15. (A) The
 798 nucleotides and amino acids are numbered on the left margin. The
 799 termination codon is indicated with asterisk (*). The Ribosomal-S19
 800 domain is shaded in blue. The peptide BjRPS15₄₅₋₆₇ is underlined in red.
 801 (B) 3D structure model of rBjRPS15. (C) 3D structure model of
 802 BjRPS15₄₅₋₆₇. (D) Exon-intron organizations of *BjRPS15* or *RPS15* in
 803 human, xenopus, zebrafish, starfish, fruit fly, octopus, Sea worm and

coral. Exons are indicated with boxes and introns with lines; blacked boxes correspond to the coding regions and empty boxes to 5' and 3' untranslated regions. The length of exons and the phases of introns are shown. The accession numbers of gene used were listed in S2 Table.

Fig 2. Transcriptional profiles of *BjRPS15* in different tissues (A), and in response to challenge with *A. hydrophila*, *E. coli*, *S. aureus* and *B. subtilis* (B) as well as LPS and LTA (C). The *EF1α* gene was chosen as internal control for normalization. (A) hc, hepatic caecum; g, gill; hg, hind-gut; nc, notochord; m, muscle; t, testis; o, ovary. (B and C) The animals were sampled at 0, 2, 4, 8, 12, 24, 48 and 72 h after the challenge, and total RNAs were extracted from the whole animals. The results shown are mean ± SEM, n = three replicates per group, and are pooled from three experiments per time point. Statistical differences between time points were assessed using unpaired Student's t-test, * $p < 0.05$, ** $p < 0.01$.

Fig 3. SDS-PAGE and Western blotting of recombinant proteins and antibacterial activity of recombinant rBjRPS15 and peptide BjRPS15₄₅₋₆₇. (A) SDS-PAGE and Western blotting of recombinant proteins rBjRPS15 and rTRX. Lane M, marker; lane 1, total cellular extracts from *E. coli* transetta (DE3) containing expression vector before induction; lane 2, total cellular extracts from IPTG-induced *E. coli* transetta (DE3) containing expression vector; lane 3, purified recombinant proteins; lane 4, Western blot of purified recombinant proteins. The proteins on the gels

were electroblotted onto PVDF membrane. After incubation with mouse anti-His-tag antibody, the membranes were incubated with the HRP-labeled goat anti-mouse IgG Ab and the bands were visualized using DAB kit. The concentrations of purified recombinant rBjRPS15 and rTRX were 100 µg/ml and 180 µg/ml respectively, and the amount of recombinant proteins loaded on the 12% SDS-PAGE gel was 20 µl. (B) Antibacterial activity of rBjRPS15 (0, 0.25, 0.5, 1 and 2 µM) and BjRPS15₄₅₋₆₇ (0, 0.625, 1.25, 2.5, 5 and 10 µM) against bacteria. The rTRX and PBS were used as control.

Fig 4. Multiple sequence alignment of the deduced amino acid sequences of BjRPS15₄₅₋₆₇ counterparts including BjRPS15₄₅₋₆₇. The identical residues among all the genes are in black.

Fig 5. TEM micrographs showing *A. hydrophila* (A) and *S. aureus* (B) that had been exposed to rBjRPS15 (2 µM), BjRPS15₄₅₋₆₇ (10 µM) or PBS (control) at 25°C for 1 h. The arrows indicated the membrane-disruptive regions of bacteria.

Fig 6. Bacterial binding activity of rBjRPS15 was revealed by Western blotting and ELISA analysis of the affinity of rBjRPS15 to the ligands LPS and LTA. (A) Binding of rBjRPS15 to the bacterial cells. Aliquots of 300 µl of bacterial suspensions (1×10^8 cells/ml) were mixed with 150 µl of 1 µM rBjRPS15 or rTRX (control), respectively. The mixtures were

incubated at 25°C for 1 h and washed three times with PBS and re-suspended in 300 µl PBS. The 20 µl bacterial suspensions were subjected to 12% SDS-PAGE gel. The bacterial suspensions on the gels were electroblotted onto PVDF membrane. After incubation with mouse anti-His-tag antibody, the membranes were incubated with the HRP-labeled goat anti-mouse IgG Ab and the bands were visualized using DAB kit. M, molecular mass standards; P, purified recombinant proteins; *Ah*, *Ec*, *Sa* and *Bs* represent *A. hydrophila*, *E. coli*, *S. aureus* and *B. subtilis* incubated with recombinant proteins, respectively. rTRX was employed as control. (B) ligand binding activity of rBjRPS15. The wells of a 96-well microplate were each coated with one of the ligands and incubated with varying concentrations of the recombinant proteins. After incubation with mouse anti-His-tag antibody, the binding was detected with HRP-labeled goat anti-mouse IgG Ab at 492 nm. Data are shown as mean ± SEM. rTRX was employed as control.

Fig 7. Bacterial membrane depolarization. Depolarization of bacteria cell membranes were detected using DiSC₃-5 (excitation, 622 nm; emission, 670 nm). rTRX and PBS were used as control.

Fig 8. Effects of rBjRPS15 and BjRPS15₄₅₋₆₇ on intracellular ROS levels. The bacteria *A. hydrophila* (A), *E. coli* (B), *S. aureus* (C) and *B. subtilis* (D) treated with rBjRPS15 and BjRPS15₄₅₋₆₇. Rosup, a compound

mixture, is able to significantly increase ROS levels in cells within 30 min. rTRX and PBS were used as control.

Fig 9. Hemolytic activity of rBjRPS15 and BjRPS15₄₅₋₆₇ to human blood cells (RBCs). Data were expressed as mean $\pm$ SEM (n = 3). The bars represent the standard error of the mean values. The symbol (***) indicates $p < 0.001$ compared with the Triton X-100 treated group. The RBCs incubated with PBS, BSA solution (100 mg/ml), and 0.1% Triton X-100 solution served as blank, negative and positive controls, respectively.

Fig 10. Western blotting and LC/MS/MS analysis. (A) Western blotting analysis of RPS15 in shrimp hemolymph, amphioxus humoral fluids, zebrafish and mouse serums. The shrimp hemolymph, amphioxus humoral fluid, zebrafish and mouse serums were run on a 12% SDS-PAGE gel (Each lane of the gel was loaded with 40 μ g of protein). The proteins on the gel were transferred to PVDF membrane. After incubation with anti-RPS15 monoclonal antibody and anti-GAPDH antibody, the membranes were incubated with a secondary HRP-labeled antibody and the bands were visualized by ECL Western blotting substrate. (B) The 40 μ g amphioxus humoral fluid loaded on the 12% SDS-PAGE and LC/MS/MS analysis was used to test if RPS15 exist in amphioxus humoral fluid. Lane M, marker; lane 1, amphioxus humoral

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905 **Supporting information**

S1 Fig. The sequence identity of BjRPS15. The amino acid identity and divergence was calculated using the Clustal W program within the MegAlign of the DNASTAR software package (version 5.0). Percent identity compares sequences directly, without accounting for phylogenetic relationships. Divergence is calculated by comparing sequence pairs in relation to the phylogeny reconstructed by MegAlign.

S2 Fig. The 3D structures of BjRPS15₄₅₋₆₇ counterparts derived from eukaryotes and prokaryotes.

S1 Table. Sequences of the primers used in this study.

S2 Table. Accession numbers of RPS15 proteins used in multiple alignment and sequence identity analysis.

A

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1  ATGGCGGACGAACAAGCTGCTCTGAAGAAGAAGCGTACCTTCAGGAAGTACACCTACCG
1  M  A  D  E  Q  A  A  L  K  K  K  R  T  F  R  K  Y  T  Y  R
60  AGGTGTCGACCTGGACCAGCTTCTGGACATGTCCAGTGAGCAGCTGATGGAAATGATGAA
21  G  V  D  L  D  Q  L  L  D  M  S  S  E  Q  L  M  E  M  M  K
120  GGCCCGTCCCCGGCGGCGCTTCTCCCGCGGCCTGAAGAGGAAACATCTCGCCCTCATCAA
41  A  R  P  R  R  R  F  S  R  G  L  K  R  K  H  L  A  L  I  K
180  GAAGCTGCGCAAGGCCAAGGAGTGTCCCGCCCTGGAGAAACCCGAGGTGGTGAAGAC
61  K  L  R  K  A  K  K  E  C  P  A  L  E  K  P  E  V  V  K  T
240  ACACTTGAGGAACACCGTCATCGTTCCTGAGATGATCGGCAGCATCGTAGCCGTCTACAA
81  H  L  R  N  T  V  I  V  P  E  M  I  G  S  I  V  A  V  Y  N
300  CGGCAAGACCTTCAACCAGGTGGAAGTTAAGCCGGAGATGATTGGGCACTACCTTGGGGA
101  G  K  T  F  N  Q  V  E  V  K  P  E  M  I  G  H  Y  L  G  E
360  GTTCTCCATCACATACAAGCCTGTGAAGCACGGCAGACCTGGTATCGGTGCCACCCACTC
121  F  S  I  T  Y  K  P  V  K  H  G  R  P  G  I  G  A  T  H  S
420  CTCACGGTTCATCCCGCTCAAGTAG
141  S  R  F  I  P  L  K  *

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Figure

Figure

Figure

RRLNRGLRRKQHSLLKRLRKAKK
 RRLNRGLRRKQNSLLKRLRKAKK
 RRLNRGLRRKQQSLLKRLRKAKK
 RREFSRGLKRKHLALIKKLRKAKK
 RKFNRLGLKRKPLALLKKLRKAKK
 RREFSRGLKRKPMALIKKLRKAKK
 RREFTRGLKRKPMALIKRLRKAKK
 RRMTRGLKRKPMALIKRLRKAKK
 RRLSRGLKRKHLALLARLQKAKK
 RREFSRGLKRKPVHLMKRLRKAKK
 RRINRLGLKRKPMGLIKKLRKAKQ
 RSLVKGLTNDQRTLMEHVRRARK
 RSLRRGLTDEQRKVLEKLRKGDG

Homo sapiens RPS15₄₃₋₆₅

Xenopus tropicalis RPS15₄₃₋₆₅

Danio rerio RPS15₄₃₋₆₅

BjRPS15₄₅₋₆₇

Acanthaster planci RPS15₄₆₋₆₈

Drosophila melanogaster RPS15₄₆₋₆₈

Octopus vulgaris RPS15₅₀₋₇₂

Capitella teleta RPS15₄₉₋₇₁

Pristionchus pacificus RPS15₄₉₋₇₁

Stylophora pistillata RPS15₄₄₋₆₆

Pseudomonas syringae RPS19₅₀₋₇₂

Nitrospirae RPS19₃₃₋₅₅

Aquificae RPS19₃₃₋₅₅

Eucaryote

Prokaryote

Figure

A

control

rBjRPS15

BjRPS15₄₅₋₆₇

B

control

rBjRPS15

BjRPS15₄₅₋₆₇

Figure

A **B**

Figure

Figure

Figure

A

B

Figure

A

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B

Figure